PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 07 March 2001 (07.03.01)	
International application No. PCT/GB00/02695	**Applicant's or agent's file reference** 10/W32314WO
International filing date (day/month/year) 13 July 2000 (13.07.00)	**Priority date** (day/month/year) 14 July 1999 (14.07.99)
Applicant RATLEDGE, Colin et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

12 January 2001 (12.01.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

A

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
MATHISEN, MACARA & CO.
The Coach House
6-8 Swakeleys Road
UB10 8BZ
UNITED KINGDOM

RECEIVED 6 MAY 2002

	Date of mailing *(day/month/year)* 16/05/2002
Applicant's or agent's file reference 10/W32494WO	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/GB 02/00495	International filing date *(day/month/year)* 04/02/2002
Applicant THE UNIVERSITY OF HULL	ACCOUNTS GT2 YES/NO

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040. Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Henriëtte Huysing-Solles

Form PCT/ISA/220 (July 1998)

PCT/GB 02/00495

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/12 C12P7/64 C12R1/89

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 01 04338 A (ANDERSON ALISTAIR JAMES ;GRANTHAM DAVID JOHN (GB); KANAGACHANDRAN) 18 January 2001 (2001-01-18) cited in the application the whole document ---	1-26
A	US 5 711 983 A (KYLE DAVID JOHN ET AL) 27 January 1998 (1998-01-27) the whole document ---	1-26
A	US 5 407 957 A (KYLE DAVID J ET AL) 18 April 1995 (1995-04-18) cited in the application the whole document ---	1-26

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 May 2002	16/05/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kools, P

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TUTTLE R C ET AL: "AN OPTIMAL GROWTH MEDIUM FOR THE DINOFLAGELLATE CRYPTHECODINIUM COHNII" PHYCOLOGIA, BLACKWELL SCIENTIFIC PUBL., OXFORD, GB, vol. 14, no. 1, 1975, pages 1-8, XP000604089 ISSN: 0031-8884 the whole document	1-26

File

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 10/W32314WO

Box No. I TITLE OF INVENTION

CULTURE OF MICROORGANISMS FOR THE SYNTHESIS OF A POLYUNSATURATED FATTY ACID

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

THE UNIVERSITY OF HULL
Cottingham Road
Hull
North Humberside HU6 7RX
United Kingdom

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

ATO BV
Bornsesteeg 59
6708 PD Wageningen
The Netherlands

This person is:

☒ applicant only

☐ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: NL

State *(that is, country)* of residence: NL

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

MATHISEN, MACARA & CO.
The Coach House
6-8 Swakeleys Road
Ickenham, Uxbridge
Middlesex UB10 8BZ
United Kingdom

Telephone No. (0)1895-678331

Facsimile No. (0)1895 676103

Teleprinter No. ---

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

14/9/2000 filed

Sheet No. 2

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

RATLEDGE, Colin
49 Church Drive
Leven
Beverley
East Yorkshire HU17 5LH
United Kingdom

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

ANDERSON, Alistair James
40 Oxenhope Road
Hull HU6 7BZ
United Kingdom

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

KANAGACHANDRAN, Kanagasooriyam
49 Trevelyan Road
London SW17 9LR
United Kingdom

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: LK

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GRANTHAM, David John
2 Malvern Crescent
Priory Road
Hull
East Yorkshire HU5 5TR
United Kingdom

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

STEPHENSON, Janet Christine
6 St. Anthony's Drive
Hedon
Hull
East Yorkshire HU12 8NT
United Kingdom

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

de SWAAF, Martin E.
Dillenburgstraat 27
3583 VD Utrecht
The Netherlands

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: NL

State *(that is, country)* of residence: NL

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SIJTSMA, Lolke
Brinkweg 18
6871 VK Renkum
The Netherlands

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: NL

State *(that is, country)* of residence: NL

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

☐ **AP** **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☐ **EA** **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ **EP** **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☐ **OA** **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

☐ **AE** United Arab Emirates
☐ **AL** Albania .....
☐ **AM** Armenia .....
☐ **AT** Austria .....
☐ **AU** Australia .....
☐ **AZ** Azerbaijan
☐ **BA** Bosnia and Herzegovina .....
☐ **BB** Barbados
☐ **BG** Bulgaria .....
☐ **BR** Brazil .....
☐ **BY** Belarus .....
☐ **CA** Canada
☐ **CH and LI** Switzerland and Liechtenstein
☐ **CN** China .....
☐ **CR** Costa Rica .....
☐ **CU** Cuba .....
☐ **CZ** Czech Republic .....
☐ **DE** Germany .....
☐ **DK** Denmark .....
☐ **DM** Dominica
☐ **EE** Estonia .....
☐ **ES** Spain .....
☐ **FI** Finland .....
☒ **GB** United Kingdom
☐ **GD** Grenada
☐ **GE** Georgia .....
☐ **GH** Ghana .....
☐ **GM** Gambia
☐ **HR** Croatia .....
☐ **HU** Hungary .....
☐ **ID** Indonesia
☐ **IL** Israel .....
☐ **IN** India .....
☐ **IS** Iceland
☐ **JP** Japan .....
☐ **KE** Kenya .....
☐ **KG** Kyrgyzstan .....
☐ **KP** Democratic People's Republic of Korea .....
☐ **KR** Republic of Korea .....
☐ **KZ** Kazakhstan .....
☐ **LC** Saint Lucia
☐ **LK** Sri Lanka
☐ **LR** Liberia
☐ **LS** Lesotho .....
☐ **LT** Lithuania
☐ **LU** Luxembourg
☐ **LV** Latvia
☐ **MA** Morocco .....
☐ **MD** Republic of Moldova .....
☐ **MG** Madagascar .....
☐ **MK** The former Yugoslav Republic of Macedonia .....
☐ **MN** Mongolia
☐ **MW** Malawi .....
☐ **MX** Mexico .....
☒ **NO** Norway
☐ **NZ** New Zealand .....
☐ **PL** Poland .....
☐ **PT** Portugal .....
☐ **RO** Romania
☐ **RU** Russian Federation .....
☐ **SD** Sudan
☐ **SE** Sweden
☐ **SG** Singapore
☐ **SI** Slovenia .....
☐ **SK** Slovakia .....
☐ **SL** Sierra Leone .....
☐ **TJ** Tajikistan .....
☐ **TM** Turkmenistan .....
☐ **TR** Turkey .....
☐ **TT** Trinidad and Tobago .....
☐ **TZ** United Republic of Tanzania
☐ **UA** Ukraine .....
☐ **UG** Uganda .....
☒ **US** United States of America .....
☐ **UZ** Uzbekistan .....
☐ **VN** Viet Nam .....
☐ **YU** Yugoslavia .....
☐ **ZA** South Africa .....
☐ **ZW** Zimbabwe .....

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

☐ .....
☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 14 July 1999	9916537.5	United Kingdom		
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): _____

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)* : ISA/

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*:
Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following number of sheets:

request	:	5
description (excluding sequence listing part)	:	23
claims	:	6
abstract	:	1
drawings	:	9
sequence listing part of description	:	–
Total number of sheets	:	44

This international application is accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: Covering letter & cheque

Figure of the drawings which should accompany the abstract: 2

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

.....
Agents for the Applicants
MATHISEN, MACARA & CO.

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA/
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference: 10/W32314WO

Applicant: THE UNIVERSITY OF HULL and ATO BV et al

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . £55 [T]

2. SEARCH FEE . . . . . £605 [S]

International search to be carried out by _____

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 44 sheets.

first 30 sheets . . . . . £264 [b1]

14 (remaining sheets) x £6 (additional amount) = £84 [b2]

Add amounts entered at b1 and b2 and enter total at B . . . . £348 [B]

Designation Fees

The international application contains 4 designations.

4 (number of designation fees payable *(maximum 8)*) x £56 (amount of designation fee) = £224 [D]

Add amounts entered at B and D and enter total at I . . . . . 572 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . --- [P]

5. TOTAL FEES PAYABLE . . . . . £1232.00 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

- ☐ authorization to charge deposit account (see below)
- ☒ cheque
- ☐ postal money order
- ☐ bank draft
- ☐ cash
- ☐ revenue stamps
- ☐ coupons
- ☐ other *(specify)*:

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ _____

☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☐ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account No. _____ Date *(day/month/year)* _____ Signature _____

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
10/W32314WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/ 02695	13/07/2000	14/07/1999
Applicant		
THE UNIVERSITY OF HULL		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   CULTURE OF CRYPTHECODINIUM COHNII FOR THE SYNTHESIS OF A POLYUNSATURATED FATTY ACID

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 2

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/02695

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P7/64 C12N1/12 A23D9/00 C12M1/36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N A23D C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, PAJ, WPI Data, FSTA, CHEM ABS Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAZHAPPILLY REMA ET AL: "Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth." JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 75, no. 3, March 1998 (1998-03), pages 393-397, XP002152068 ISSN: 0003-021X	1-6, 20-39
Y	page 396, right-hand column; tables 1-3	7-19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2000	22/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Devijver, K

4

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	DU PREEZ J C ET AL: "Production of gamma-linolenic acid by Mucor circinelloides and Mucor rouxii with acetic acid as carbon substrate." BIOTECHNOLOGY LETTERS, vol. 17, no. 9, 1995, pages 933-938, XP000960382 ISSN: 0141-5492 page 933 -page 936; figures 1,2; table 2 ---	7-19
A	US 5 407 957 A (KYLE DAVID J ET AL) 18 April 1995 (1995-04-18) cited in the application the whole document -----	1-39

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/02695

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5407957 A	18-04-1995	AT 165212 T	15-05-1998
		AU 660162 B	15-06-1995
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		US 5397591 A	14-03-1995

Eicosapentaenoic Acid and Docosahexaenoic Acid Production Potential of Microalgae and Their Heterotrophic Growth

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ABSTRACT: Twenty microalgal strains were investigated in photoautotrophic flask cultures for their potential for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) production. The highest EPA proportion (% of total fatty acids) was produced by *Monodus subterraneus* UTEX 151 (34.2%), followed by *Chlorella minutissima* UTEX 2341 (31.3%) and *Phaeodactylum tricornutum* UTEX 642 (21.4%). The highest DHA proportion (% of total fatty acids) was obtained in *Crypthecodinium cohnii* UTEX L1649 (19.9%), followed by *Amphidinium carterae* UTEX LB 1002 (17.0%) and *Thraustochytrium aureum* ATCC 28211 (16.1%). Among the 20 strains screened, the EPA yield was high in *M. subterraneus* UTEX 151 (96.3 mg/L), *P. tricornutum* UTEX 642 (43.4 mg/L), *Chl. minutissima* UTEX 2341 (36.7 mg/L), and *Por. cruentum* UTEX 161 (17.9 mg/L) owing to their relatively high biomass concentrations. The DHA yield was high in *C. cohnii* UTEX L1649 (19.5 mg/L) and *A. carterae* UTEX LB 1002 (8.6 mg/L). Heterotrophic growth of these 20 microalgae was also tested on two different carbon sources, acetate and glucose. All microalgae except *Nannochloropsis oculata* UTEX LB 2164 showed growth on glucose (5 g/L) under heterotrophic conditions. Twelve of them could grow heterotrophically when acetate (1 g/L) was used as their sole carbon and energy source.

JAOCS 75, 393–397 (1998).

KEY WORDS: Docosahexaenoic acid, eicosapentaenoic acid, n-3 fatty acids, heterotrophic, microalgae, photoautotrophic.

Recent clinical and epidemiological studies have indicated that long-chain n-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), are important in the treatment of atherosclerosis, cancer, rheumatoid arthritis, psoriasis, and diseases of old age, such as Alzheimer's disease and age-related macular degeneration (1,2). The eicosanoids, such as prostaglandin, prostacyclin and leukotriene, derived from n-3 polyunsaturated fatty acids are also important in infant development, modulatory vascular resistance, and wound healing (2,3).

Fish oil is the richest source of n-3 fatty acids and contains up to 30% of DHA and EPA (4). At present, fish oil is used for the commercial production of EPA and DHA. However, there are some limitations to using fish oil as a source of n-3 fatty acids, such as peculiar taste, odor and stability problems. Purification costs of these fatty acids from fish oil are also high. Because fish obtain n-3 fatty acids from zooplankton, which consume algae, research has become focused on developing a commercially feasible technology to produce n-3 fatty acids directly from microalgae (5). Several attempts have been made recently to develop a commercial heterotrophic process for n-3 fatty acid production because photoautotrophic production is limited by the cells' mutual shading and the consequent increase in downstream processing cost (6). Heterotrophic production of DHA by the dinoflagellate *Crypthecodinium cohnii*, grown in fermentors, has just been commercialized by Martek Biosciences in Maryland (7). The advantages and limitations of heterotrophic growth of microalgae have been recently reviewed (8).

Although the fatty acid composition and growth of microalgae may be influenced by nutrients and environmental conditions, the fatty acid production potential of microalgae depends highly on primary strain selection for maximal fatty acid production. The industrial heterotrophic production potential of microalgae mainly depends on the ability of the selected strains to grow heterotrophically with good fatty acid compositions and yields. The aim of the present study was to screen and identify microalgal strains with high EPA and DHA yields and to test their heterotrophic growth potential.

MATERIALS AND METHODS

Organisms. The microalgae were obtained from culture collections (i.e., UTEX, ATCC, and CSIRO) as shown in Table 1.

Photoautotrophic growth. The media used for culturing these microalgae were prepared according to recommendations by the culture collections (Table 1). All media were inoculated with a 5% inoculum of exponentially growing cells. The microalgae were grown in 250-mL flasks that contained 150 mL medium in batch mode at 25°C with intermittent shaking and continuous illumination (60 μE/m^2 s).

Heterotrophic growth. The same media recommended by the culture collections were used. An inoculum (5%) of exponentially growing cells was used for inoculation. All microalgae were grown separately on glucose (5 g/L) and acetate (1 g/L) in 250-mL flasks with 150 mL medium in batch mode in

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TABLE 1
Growth of Microalgae Under Photoautotrophic and Heterotrophic Conditions[a]

Microalgae	Medium[b,c]	Culture time[d] (d)	Biomass concentration[d] (mg/L)	On glucose[e] (5 g/L)	On acetate[e] (1 g/L)
A. carterae UTEX LB 1002	Erd-Schreiber	23	499	+ + +	–
Amphidinium sp. CSIRO CS-259	G2	23	330	+ + +	+ +
Chaetoceros calcitrans CSIRO CS-178	f/2	20	409	+	+
Chlorella minutissima UTEX 2219	Proteose	10	894	+	+
Chl. minutissima UTEX 2341	Artificial seawater	10	995	+	+
Chröomonas salina CSIRO CS-174	fE	23	157	+	–
Crypthecodinium cohnii UTEX L1649	Porphyridium	10	1510	+ + + +	+ + +
Cryptomonas sp. UTEX LB 2423	ES-Enriched seawater	25	316	+ +	–
Isochrysis galbana UTEX LB 987	Soil + seawater	20	871	+	+
Monodus subterraneus UTEX 151	Proteose	10	2566	+	+
Nannochloropsis oculata UTEX LB 2164	Erd-Schreiber	25	345	-	–
Pavlova lutheri UTEX LB 1293	Erd-Schreiber	25	235	+	–
P. lutheri ATCC 50092	Hesnw	25	368	+	–
Phaeodactylum tricornutum UTEX 642	LDM	10	1934	+ + +	+
Porphyridium cruentum UTEX 161	Porphyridium	10	964	+ +	+
Por. purpureum CSIRO CS-25	f/2	23	456	+ +	–
Prorocentrum minimum UTEX LB 1995	Erd-Schreiber	20	398	+	–
Schizochytrium aggregatum ATCC 28209	By+	20	488	+ + +	+ +
Thalassiosira pseudonana CSIRO CS-173	G2	25	153	+ +	+
Thraustochytrium aureum ATCC 28211	By+	20	101	+ + +	+ +

[a]Culture collections: UTEX—The Culture Collection of Algae at The University of Texas at Austin, Texas; ATCC—American Type Culture Collection, Rockville, Maryland; CSIRO—CSIRO Marine Laboratories, Hobart, Tasmania, Australia.
[b]Medium : Erd-Schreiber, proteose, artificial seawater, porphyridium, ES-enriched seawater, soil + seawater and LDM (9); Hesnw and By+ (By+ medium was prepared without glucose) (10); f/2, G2 and fE medium compositions were provided by CSIRO Marine Laboratories.
[c]Artificial seawater was used instead of natural seawater in all media.
[d]Photoautotrophic growth.
[e]Heterotrophic growth: + poor growth; + + moderate growth; + + + good growth, + + + + excellent growth.

darkness. The culture temperature was maintained at 25°C with continuous shaking at 200 rpm in an orbital shaker.

Determination of biomass. The cell dry weight concentration was determined according to the method previously reported by Chen and Johns (11).

Extraction and purification of lipids. Lipids were extracted by modified procedures of Bligh and Dyer (12) and Chen and Johns (11). The cells were first homogenized with chloroform/methanol/water (1:2:0.8, vol/vol/vol) for 2 min. An equal volume of chloroform and water was then added, to bring the final ratio of the mixture to 2:2:1.8, and the mixture was then homogenized for 1 min. The chloroform layer (lower) with the lipids was then separated, and the alcoholic layer (upper), which contained the residues, was reextracted twice with methanol/chloroform (2:1, vol/vol).

The chloroform layers were combined and subjected to a "Floch wash" to remove all nonlipid contaminants (13). The mixture was washed with one-fourth of the volume of 0.88% (wt/vol) potassium chloride, followed by methanol/saline solution (1:1, vol/vol). The purified chloroform layer was then evaporated to dryness under N_2 and weighed to get the total lipid content.

Fatty acid analysis. The lipids were then transesterified by alkali catalysis (13). The lipid samples were dissolved in benzene (up to 100 mg in 2 mL). An appropriate amount of internal standard, C17:0 (heptadecaenoic acid), was added at this stage. Sodium methoxide (0.5 M) in anhydrous methanol (2 mL) was added, and the mixture was incubated at 50°C for 10 min. Glacial acetic acid (0.1 mL), followed by water (5 mL), was added, the esters were extracted with hexane (10 mL), and the hexane layers were then concentrated under N_2.

The purity of fatty acid methyl esters was checked by thin-layer chromatography (13). The algal fatty acid methyl esters were run along with standard fatty acid methyl esters (Sigma Chemical Co., St. Louis, MO) in hexane/diethyl ether (9:1, vol/vol) as the mobile phase. The fatty acid methyl esters were then used for gas-chromatographic analysis in an HP-6890 GC (Hewlett-Packard, Palo Alto, CA), equipped with a flame-ionization detector and a Supelco (Bellefonte, PA) Omegawax™ 250 capillary column (30 m × 0.25 mm). The column and detector temperatures were kept at 210 and 260°C, respectively. Nitrogen was used as the carrier gas, and the flow rate was kept at 2 mL/min. The injector was kept at 250°C, with an injection volume of 3 μL under splitless injection mode. Authentic standards (Sigma Chemical Co.) were employed for the identification of fatty acids. The fatty acids were identified by comparison of relative retention times and by calculation of equivalent chainlengths. The fatty acid contents were determined by comparing their peak areas with that of the internal standard (C17:0).

RESULTS AND DISCUSSION

Among the 20 microalgae tested under photoautotrophic con-

ditions, six showed good growth after 10 d, while the rest required 20–25 d to produce significant biomass. The growth times, media, and biomass concentrations are shown in Table 1. The fatty acid composition and the EPA and DHA yields, obtained from each species, are shown in Tables 2 and 3, respectively.

Monodus subterraneus UTEX 151, which is a freshwater strain, produced the highest cell dry weight concentration (Table 1). This alga also had the highest EPA proportion, EPA content, and EPA yield (Tables 2 and 3). Increased EPA proportion and content at a higher light intensity (90 $\mu E/m^2$ s) and at a lower temperature (20°C) were previously reported in *M. subterraneus* (14). The slight reduction in EPA proportion and content may be due to the higher temperature (25°C) and different culture conditions (e.g., light intensity) used. The inverse relation of temperature to EPA content in *M. subterraneus* was also reported by Iwamoto and Sato (15). *Chlorella minutissima* UTEX 2341 also had a high EPA proportion, content, and yield (Tables 2 and 3), although they were lower than for *M. subterraneus*. Even though *Phaeodactylum tricornutum* UTEX 642 had lower EPA proportion and content than *Chl. minutissima* UTEX 2341, the EPA yield was higher owing to higher biomass concentrations achieved. *Porphyridium cruentum* UTEX 161 also had high EPA proportion, content, and yield.

Crypthecodinium cohnii UTEX L 1649 had the highest DHA proportion and yield among all 20 microalgal strains. *Amphidinium carterae* UTEX LB 1002 also had a high DHA proportion and yield. Although the DHA proportion and content of *A. carterae* UTEX LB 1002 were comparable to *Cr. cohnii* UTEX L 1649, the yield was much lower due to lower attainable biomass concentration (Table 1). *Thraustochytrium aureum* ATCC 28211 also had a relatively high DHA proportion and content, but the yield was low owing to poor biomass concentration (only 0.1 g/L after 23 d of cultivation). The DHA proportion and content of *Thr. aureum* ATCC 28211 were both lower than those of another *Thr. aureum* strain (ATCC 34304) reported by Bajpai *et al.* (16).

Although the EPA yield (17.9 mg/L) was low in *Por. cruentum* UTEX 161, the EPA yield (Table 3) obtained from *Chl. minutissima* UTEX 2341 and *Ph. tricornutum* UTEX 642 were high compared to the screening results obtained from the same strains by Yongmanitchai and Ward (17) owing to higher biomass concentrations. This is probably due to the different growth conditions used. The EPA contents (% of biomass) of *Chl. minutissima* UTEX 2341 and *Por. cruentum* UTEX 161 were low compared to the results of Yongmanitchai and Ward (17). This may be due to the effects of culture conditions, especially temperature and aeration. In their experiments, a lower temperature (20°C) and higher aeration rate (75 mL/min) were employed. Among the 20 microalgae tested, *Amphidinium* sp. CSIRO CS-174, *Chaetoceros calcitrans* CSIRO CS-178, *Cryptomonas* sp. UTEX LB 2423, *Isochrysis galbana* UTEX LB 987, *Pavlova lutheri* ATCC 50092, *Schizochytrium aggregatum* ATCC 28209, and *Thalassiosira pseudonana* CSIRO CS-173 also contained significant proportions of EPA (Table 2). The EPA and DHA contents were not reported in the specific strains of microalgae used in this experiment except in *A. carterae* UTEX LB 1002, *Chl. minutissima* UTEX 2219, *Chl. minutissima* UTEX 2341, *M. subterraneus* UTEX 151, *Ph. tricornutum* UTEX 642, and *Por. cruentum* UTEX 161 (Tables 2 and 3).

The high accumulation of arachidonic acid (ARA, C20:4n-6) with EPA or DHA is disadvantageous because ARA can cause deleterious health effects and problems in EPA recovery (17–19). The ARA content was relatively low

TABLE 2
Fatty Acid Compositions of Microalgae (% of total fatty acids)[a]

Microalgae	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:5	22:6
A. carterae	5.4	2.1	30.9	7.1	10.5	0.3	5.6	3.1	1.6	15.1	1.3	17.0
Amphidinium sp.	7.8	0.6	28.0	23.7	6.7	8.5	4.7	0.8	1.1	16.3	1.8	0.0
Cha. calcitrans	7.0	1.4	27.5	26.5	7.2	0.1	4.9	1.0	1.1	18.8	4.5	0.0
Chl. minutissima (UTEX 2219)	11.4	2.7	30.5	26.8	4.9	3.3	0.3	10.3	2.6	3.3	3.9	0.0
Chl. minutissima (UTEX 2341)	0.7	4.7	11.9	15.0	7.8	12.0	6.3	4.6	5.6	31.3	0.1	0.0
Chr. salina	12.0	5.1	22.6	21.9	2.0	0.3	19.7	3.1	4.1	8.2	1.0	0.0
Crypth. cohnii	4.4	16.9	20.6	22.6	9.0	0.3	2.3	1.1	0.9	0.0	2.0	19.9
Cryptomonas sp.	7.9	1.0	21.7	17.1	15.6	3.2	1.4	1.8	2.8	16.6	0.7	10.2
I. galbana	3.5	2.7	28.2	19.3	6.7	0.3	4.0	12.9	1.2	16.6	1.0	3.6
M. subterraneus	0.1	12.7	18.7	10.1	0.9	5.4	2.4	0.4	13.7	34.2	1.4	0.0
N. oculata	3.0	3.2	28.8	14.5	4.1	3.3	10.0	14.1	5.1	5.5	1.3	7.1
Pa. lutheri (UTEX LB1293)	8.6	7.6	22.8	4.6	11.5	0.6	20.3	1.4	6.8	3.9	4.2	7.7
Pa. lutheri (ATCC 50092)	13.4	3.0	10.7	3.2	12.6	7.5	9.2	9.0	5.4	14.3	5.9	5.8
Ph. tricornutum	6.6	2.7	14.0	33.9	0.0	7.2	4.0	0.7	3.2	21.4	5.8	0.5
Por. cruentum	0.3	17.5	35.5	8.5	0.0	0.0	1.3	0.0	17.2	19.7	0.0	0.0
Por. purpureum	0.1	2.8	37.9	1.4	13.7	4.4	8.1	8.6	5.8	6.7	10.5	0.0
Pro. minimum	6.3	2.9	33.2	2.8	17.8	0.3	6.1	10.6	2.7	8.7	2.7	5.9
S. aggregatum	2.6	2.7	15.3	18.6	8.0	14.7	15.1	0.6	7.2	15.7	0.0	0.0
Tha. pseudonana	5.8	0.8	31.7	17.6	2.0	5.4	2.9	0.6	9.2	10.8	6.7	6.5
Thr. aureum	0.3	8.9	8.5	5.4	6.7	7.8	10.5	15.2	12.1	4.5	4.0	16.1

[a]See Table 1 for abbreviations.

TABLE 3
Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Yields of Microalgae[a]

Microalgae	EPA of biomass (% w/w)	DHA of biomass (% w/w)	EPA yield (mg/L)	DHA yield (mg/L)
A. carterae UTEX LB 1002	1.5	1.7	7.7	8.6
Amphidinium sp. CSIRO CS-259	1.2	0.0	4.0	0.0
Cha. calcitrans CSIRO CS-178	1.0	0.0	4.2	0.0
Chl. minutissima UTEX 2219	0.3	0.0	2.7	0.0
Chl. minutissima UTEX 2341	3.7	0.0	36.7	0.0
Chr. salina CSIRO CS-174	0.4	0.0	0.6	0.0
Crypth. cohnii UTEX L1649	0.0	1.3	0.0	19.5
Cryptomonas sp. UTEX LB 2423	1.0	0.6	3.2	2.0
I. galbana UTEX LB 987	0.9	0.2	8.0	1.7
M. subterraneus UTEX 151	3.8	0.0	96.3	0.0
N. oculata UTEX LB 2164	0.6	0.7	2.0	2.6
Pa. lutheri UTEX LB 1293	0.2	0.5	0.6	1.1
Pa. lutheri ATCC 50092	0.8	0.3	3.0	1.2
Pha. tricornutum UTEX 642	2.2	0.06	43.4	1.08
Por. cruentum UTEX 161	1.9	0.0	17.9	0.0
Por. purpureum CSIRO CS-25	0.8	0.0	3.7	0.0
Pro. minimum UTEX LB 1995	0.6	0.4	2.3	1.5
S. aggregatum ATCC 28209	1.2	0.0	6.1	0.0
Tha. pseudonana CSIRO CS-173	0.5	0.3	0.8	0.5
Thr. aureum ATCC 28211	0.3	1.0	0.3	1.0

[a]See Table 1 for abbreviations.

in all 20 microalgae except in *Por. cruentum* UTEX 161, which is a well-known species for ARA or EPA production, depending on the environmental conditions used (19–21). Optimization of growth conditions, such as temperature and salinity, can reduce ARA content and enhance EPA accumulation in *Por. cruentum* (20,21).

Out of the 20 microalgae screened, *M. subterraneus* UTEX 151, *Ph. tricornutum* UTEX 642, *Chl. minutissima* UTEX 2341, *Por. cruentum* UTEX 161, and *Crypth. cohnii* UTEX L1649 yielded significant amounts of EPA or DHA (18–96 mg/L) (Table 3). When considering the individual production of EPA and DHA attributable to their different nutritional and pharmacological importance, it is desirable to select strains that contain less EPA for DHA production or vice versa, to minimize problems associated with downstream processing (17). For example, *Crypth. cohnii* UTEX L 1649 was the best potential strain for DHA production, which yielded more than 19 mg/L of DHA, without production of EPA (Table 3). In contrast, *A. carterae* UTEX LB 1002 yielded about equal amounts of EPA (7.7 mg/L) and DHA (8.6 mg/L).

The screening results indicated that *M. subterraneus* UTEX 151 was the alga with the best potential for EPA production, with high biomass, high growth rate, high EPA, and absence of DHA. *Crypthecodinium cohnii* showed characteristics for good DHA production, such as high biomass, high growth rate, high DHA content, and complete absence of EPA.

The EPA and DHA proportions and contents of some strains obtained in this study are lower than those reported by other investigators. This is probably due to the effects of culture conditions, such as temperature and aeration. The extreme sensitivity of unsaturated fatty acid composition to growth temperature, to maintain membrane fluidity, is reported by many researchers (19,22,23). Fatty acid unsaturation due to changes in growth temperature is associated with dissolved oxygen concentration in batch culture (24) and also depends on the availability of intracellular molecular oxygen (25). In this screening study, aeration was not provided (only intermittent shaking), and because the optimal temperature for maximal fatty acid unsaturation may vary in each species, the temperature used (25°C) might not be optimal.

The growth of microalgae on glucose and acetate under heterotrophic conditions was also investigated (Table 1). All microalgae except *Nannochloropsis oculata* UTEX LB 2164 showed heterotrophic growth when glucose was used as the sole carbon and energy source. Twelve of them could grow heterotrophically on acetate.

Out of the 20 microalgae tested (Table 1), *Crypth. cohnii* UTEX L1649 showed excellent heterotrophic growth on glucose. *Amphidinium carterae* UTEX LB 1002, *Amphidinium* sp. CSIRO CS-259, *Ph. tricornutum* UTEX 642, *S. aggregatum* ATCC 28209, and *Thr. aureum* ATCC 28211 also showed good heterotrophic growth on glucose.

When acetate was used as the sole carbon and energy source, only *Crypth. cohnii* UTEX L 1649 showed good heterotrophic growth, while several other strains, *Amphidinium* sp. CSIRO CS-259, *S. aggregatum* ATCC 28209 and *Thr. aureum* ATCC 28211, showed moderate growth on acetate. Table 1 shows that the preferred organic substrate for heterotrophic growth is glucose for all strains tested. We are not sure whether this difference is due to the high concentration of acetate (1 g/L) used because acetate might be inhibitory to microalgae above certain concentrations (26,27). An acetate concentration of 0.4 g/L or more was found to be toxic to a green microalga, *Chlamydomonas reinhardtii*, grown heterotrophically.

Although a decrease in fatty acid unsaturation was reported in heterotrophic cultures of microalgae owing to the accumulation of triglycerides, some recent studies indicated that fatty acid unsaturation might increase or decrease under heterotrophic conditions, depending on the algal strains employed (28,29). Studies are going on in this laboratory to further identify potential heterotrophic EPA- and DHA-producing microalgae and to develop a heterotrophic high cell density process for the production of n-3 polyunsaturated fatty acids.

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PRODUCTION OF GAMMA-LINOLENIC ACID BY *MUCOR CIRCINELLOIDES* AND *MUCOR ROUXII* WITH ACETIC ACID AS CARBON SUBSTRATE

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SUMMARY

The production of gamma-linolenic acid (GLA) by *Mucor circinelloides* CBS 203.28 and *M. rouxii* CBS 416.77 in fed-batch cultures operated in pH-stat mode with acetic acid as carbon substrate and titrant compared favourably with the performance of *M. circinelloides* in batch culture on glucose. On acetic acid *M. circinelloides* accumulated up to 39.8 mg GLA/g biomass, with a crude oil content of 28% containing 91% neutral lipids. The GLA content of the neutral lipid fraction was 15.6%.

INTRODUCTION

Gamma-linolenic acid (GLA), an 18:3 ω6 polyunsaturated fatty acid, is used in the treatment of various illnesses such as rheumatoid arthritis (Jantti *et al.*, 1989), multiple sclerosis (Barber, 1988), atopic eczema (Scott, 1989), schizophrenia (Horrobin, 1979), premenstrual syndrome (Khoo *et al.*, 1990) and, being a precursor of prostaglandins (biologically active lipid hormones), it is also of pharmaceutical interest (Weete and Ghandi, 1992). Moulds of the Zygomycetes, especially the Mucorales, are known to accumulate GLA in the mycelium (Shaw, 1965). Currently the only commercial plant for the production of GLA of fungal origin, using *Mortierella*, is in Japan (Ratledge, 1993).

Acetic acid is a feedstock commonly used in the chemical industry (Shreve, 1967). Aqueous acetic acid solutions are also a by-product of certain industrial synthetic processes (Cama and Edwards, 1970). Because it is a cheaper feedstock than the carbohydrate substrates typically used in industrial fermentation processes, acetate has potential as carbon feedstock for microbial processes. Unfortunately, acetic acid is inhibitory to microbial growth (Sestáková, 1979; Noda *et al.*, 1982; Moon, 1983). Thus, in this investigation on the production of GLA by two *Mucor* species grown on acetic acid, fed-batch cultures operated in a pH-stat mode with acetic acid as titrant and carbon source were used to facilitate a constant low concentration of acetic acid throughout the cultivation. A nitrogen limitation, known to enhance lipid accumulation (Moreton, 1988; Ratledge, 1991), was also imposed on the culture. Recently we reported on the use of this procedure to demonstrate that a certain strain of *M. circinelloides* produced both GLA and a cocoa butter equivalent from acetic acid (Kock and Botha, 1994; Roux *et al*, 1994). In the present study, however, the focus was on evaluating selected strains of *Mucor* in terms of GLA production with acetic acid as carbon and energy source.

MATERIALS AND METHODS

Microorganisms. *Mucor circinelloides* CBS 203.28 (type strain) and *Mucor rouxii* CBS 416.77 were from the culture collection of this department. These moulds were maintained on 4% Sabouraud dextrose (SAB) agar at 4°C.

Preparation of inocula. A spore inoculum was prepared by streaking out spores from fresh SAB-agar slants onto a Petri dish with SAB agar, incubating for 2 to 3 days at 30°C and subsequently washing off the spores with a 0.05 M K_2HPO_4 buffer solution at pH 8.0, 1 ml of which was used to inoculate a second Petri dish. After further incubation for 2 to 3 days, the spores were washed off with the above buffer solution containing 0.1% Tween 80, which prevented the spores from clumping, and used to inoculate the bioreactor to an initial concentration of 120 to 180×10^3 spores/ml. Spore counts were done in Fast-read 10 counting chambers (Davies Diagnostics, Randburg).

A vegetative inoculum for bioreactor cultivation on acetic acid was prepared by inoculating a 1 l flask containing 900 ml medium at an initial pH of 5.5 with about 1×10^5 spores/ml, prepared from the first Petri dish as described above. The flask, fitted with a cotton wool plug, was stirred with a magnetic bar at 900 r/min for vortex aeration and incubated for 24 h at 30°C (for cultures on acetic acid). The medium comprised (per litre distilled water): KH_2PO_4, 7 g; Na_2HPO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; yeast extract, 3 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; KNO_3, 3.3 g and glucose, 30 g., adjusted to pH 5.5. The inoculum used for bioreactor cultivation on glucose was prepared in a similar fashion as above, but starting with mycelium and spores from 3-day old YM slants containing yeast extract, malt extract and glucose, and the KNO_3 in the medium was replaced with 3 g sodium glutamate. An inoculation density of 0.4 to 0.68 g/l was used.

Bioreactor cultivation. A 14 l stirred tank reactor (STR) (Chemap 3000, Chemap AG, Volketswil), fitted with three disk turbine impellers and using a working volume of 10.9 l, was used for cultivations on acetic acid. The medium contained (per litre distilled water): citric acid, 0.16 g; NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1.88 g; $CaCl_2 \cdot 2H_2O$, 0.03 g; yeast extract, 0.5 g and trace elements according to du Preez and van der Walt (1983). When a spore inoculum was used, 0.5 g glucose was added to facilitate germination. After *in situ* sterilisation of the reactor, a sterile solution of sodium acetate was added to give an initial acetic acid concentration of 2 g/l and the medium adjusted from pH 5.6 to pH 7 with 7 M NaOH. After allowing 6 h for spore germination, the medium was adjusted to pH 6 with 10 N H_2SO_4 and controlled at this pH value by automatic titration with concentrated acetic acid diluted 1:1 (by volume) with distilled water. When a vegetative inoculum was used, the medium was adjusted to pH 6 with 7 M NaOH immediately after inoculation. The dissolved oxygen tension (DOT) in the medium was maintained at or above 20% of saturation by manually increasing the air flow rate from 9 to 11 l/min and by automatic control of the stirrer speed in the range of 200 to 550 r/min.

Batch cultivations on glucose were conducted in a 5 l STR, using a 4 l working volume, equipped with three open turbine impellers and controlled at pH 5.5 with 2 M KOH. A constant stirrer speed of 700 r/min at an aeration rate of 2 l/min maintained the DOT above 60% of saturation. The medium was as above, but with no citric acid, 1.7 g $(NH_4)_2SO_4$/l instead of NH_4Cl, 1 g yeast extract/l and 50 g glucose/l.

Analytical procedures. The biomass concentration was gravimetrically determined in duplicate by filtration of 10 ml aliquots through pre-dried and weighed glass microfibre filters (Whatman GF/D or GF/A), washing with distilled water and drying at 105°C. Acetic acid was determined with a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionisation detector and a 30 m x 0.53 mm Nukol™ fused silica capillary column (Supelco Inc., Sulco Park, Bellefonte), using nitrogen gas as carrier (10 ml/min) and linear temperature programming at a rate of 6°C/min up to 175°C following an initial 3 min period at 100°C. Each sample was acidified with formic acid (4% v/v, final concentration) prior to injection of a 1 µl sample. Nitrogen concentrations were determined by the indophenol method (Chaney and Marbach, 1962). Biomass was harvested by filtration, frozen for 24 h at -20°C, lyophilized and the lipids analysed according to Kock and Ratledge (1993). The fatty acids were determined by injection of 1 µl samples into a Varian

3300 gas chromatograph equipped with a flame ionisation detector and a 30 m x 0.75 mm glass capillary Supelco Wax 10 column (Supelco Inc., Sulco Park, Bellefonte), using nitrogen gas as carrier (5 ml/min) and linear temperature programming at a rate of 3°C/min up to 245°C following an initial 3 min period at 145°C.

Determination of oxygen solubility and uptake. The solubility of oxygen in the medium was determined according to the method of Käppelli and Fiechter (1981). The oxygen uptake rates of *M. circinelloides* on acetic acid were determined by interrupting aeration and recording the decrease in DOT, measured with an Ingold polarographic electrode, at 1 s intervals with an electronic data logger at a low stirrer speed of 150 to 300 r/min to minimize surface aeration. The critical dissolved oxygen concentration (defined as that value below which the respiration rate is a function of the dissolved oxygen concentration) was determined from plots of the DOT versus time at the point where the trace deviated from linearity. The solubility of oxygen in distilled water, correcting for temperature and barometric pressure, was used in the calculations.

RESULTS AND DISCUSSION

The procedures used for the production of an actively growing vegetative inoculum resulted in the formation of a population consisting of yeast-like cells as well as short, sparsely branched mycelial fragments. Ethanol was also produced. Dimorphism in certain *Mucor* species is well documented and the transition to a yeast-like morphology, i.e. spherical multipolar budding cells, is induced by microaerobiosis or by microaerobiosis in combination with a high CO_2 level or glucose concentration (Bartnicki-Garcia, 1963, 1968; Bartnicki-Garcia and Nickerson, 1962; Ruiz-Herrera, 1993).

The method of Käppeli and Fiechter (1981) did not indicate a significant difference between the oxygen solubility in distilled water and in the culture medium containing acetic acid. This was probably because the medium contained only low concentrations of solutes and not, as with most culture media, a high concentration of sugar. Therefore, the oxygen solubility value for distilled water was used for converting the readings obtained with the dissolved oxygen probe to oxygen concentration. With *M. circinelloides* the highest values of the specific rate of oxygen uptake (qO_2, 170 mg/g biomass.h) and the critical dissolved oxygen concentration (C_{crit}, 0.98 mg O_2/l, equivalent to a DOT value of 15% of air saturation) were obtained during the exponential growth phase (Table 1). These values subsequently decreased with the decrease in growth rate. The C_{crit} values were in close agreement with those reported for another strain of *M. circinelloides* grown under comparable conditions (Roux *et al.*, 1995).

In eukaryotic microorganisms the introduction of a double bond into a fatty acyl chain involves a mono-oxygenase catalysed reaction requiring NADPH and molecular oxygen (Rattray *et al.*, 1975). Thus, oxygen availability can be expected to affect desaturation (and, therefore, also GLA synthesis), as was reported by others (Choi *et al.*, 1982; Davies *et al.*, 1990; Roux *et al.*, 1995). Because the data of Roux *et al.* (1995) suggested that DOT values above the C_{crit} value for respiration may affect desaturation in *M. circinelloides*, the cultivations on acetic acid were, therefore, conducted at a constant DOT of 20% saturation.

The fed-batch, pH-stat mode of cultivation enabled the acetic acid concentration in the culture to be maintained at about 2 g/l to minimize inhibitory effects. Typical results are presented in Table 2. Not surprisingly, an actively growing vegetative inoculum as opposed

Table 1. The specific oxygen uptake rate (qO_2) and critical dissolved oxygen concentration (C_{crit}), also expressed in terms of the corresponding percentage of saturation, of *M. circinelloides* at different time intervals during fed-batch cultivation on acetic acid. The mean values of three to four determinations and the corresponding standard deviation of the mean (in brackets) are given.

Time (h)	qO_2	C_{crit}	
	mg O_2/g.h	mg O_2/l	% of saturation
7	169.8 (±1.41)	0.983 (±0.08)	15.2 (±1.29)
24	46.7 (±2.07)	0.796 (±0.12)	12.3 (±1.86)
55	24.2 (±3.04)	0.460 (±0.08)	7.2 (±1.30)

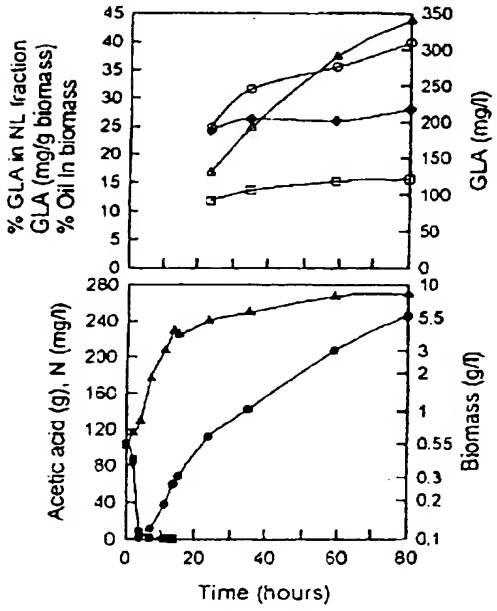

Figure 1. Fed-batch cultivation profile of *Mucor circinelloides* on acetic acid concentration of 2 g/l acetic acid using a vegetative inoculum. Symbols: ● Acetic acid titrated; ■ nitrogen; ▲ biomass; □ GLA content of the neutral lipid (NL) fraction; ○ GLA content of the biomass; △ mg GLA/l culture; ◆ crude oil content of the biomass.

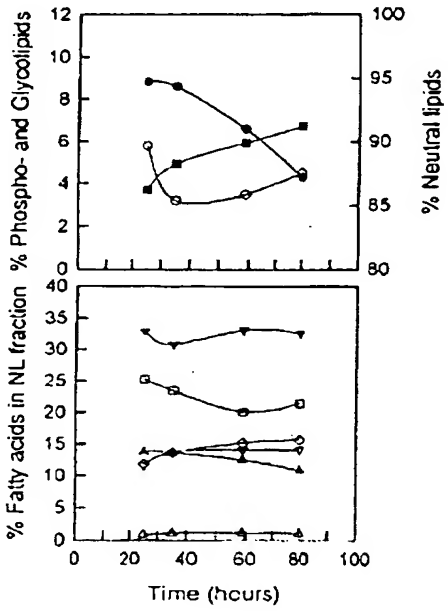

Figure 2. Changes in lipid composition of the crude oil and fatty acid profile of the neutral lipid fraction (NL) of *Mucor circinelloides* grown on acetic acid, using the vegetative inoculum. Symbols: 16:0 (□); 16:1 (△); 18:0 (▲); 18:1 (▼); 18:2 (▽); 18:3 (◇); neutral lipid (■), glycolipid (●) and phospholipid (○) fraction.

Table 2. Comparison of growth, lipid and gamma-linolenic acid (GLA) accumulation by two *Mucor* species grown in fed-batch cultures with acetic acid as carbon source and in batch culture with glucose.

Parameters	*Mucor rouxii*		*Mucor circinelloides*		
	Acetate[1]	Acetate[2]	Acetate [1]	Acetate[2]	Glucose[3]
Cell concentration (g/l)	6.79	8.53	7.85	8.53	11.0
$Y_{x/s}$	0.25	0.31	0.27	0.30	0.25
μ_{max} (h^{-1})	0.30	0.20	0.19	0.25	0.28
GLA content (% of NL)	11.8	13.7	13.5	15.6	16.3
GLA content (mg/g biomass)	30.4	34.0	36.6	39.8	35.5
GLA concentration (mg/l culture)	206	290	287	339	390
Y_{GLA} (g GLA/g substrate)	0.008	0.01	0.01	0.012	0.01
Q_p (mg GLA/l culture.h)	2.54	2.78	2.19	3.74	4.59
% Crude oil in biomass	26.3	27.6	31.3	28.0	24.2
% NL in crude oil	90.8	89.8	86.7	91.2	90.0

Y_{GLA} GLA yield coefficient on carbon substrate assimilated.
$Y_{x/s}$ Biomass yield on carbon substrate assimilated.
μ_{max} Maximum specific growth rate.
Q_p Overall volumetric rate of GLA production.
NL Neutral lipid fraction of crude oil.
[1] Spore inoculum, 100 h cultivation time.
[2] Yeast-like vegetative inoculum, 80 h cultivation time.
[3] Yeast-like vegetative inoculum, 85 h cultivation time.

to a spore inoculum gave the better performance in terms of most of the parameters, probably because of the time required for spore germination. The overall performance of *M. circinelloides* under these conditions was consistently slightly better than that of *M. rouxii*. The best results obtained with *M. circinelloides* are shown in Table 2, where the GLA content exceeded 15% of the NL fraction and amounted to 39.8 mg/g biomass, which had a crude oil content of 28% containing 91% neutral lipids. The filamentous nature of the fungi rendered the taking of homogeneous samples required for the accurate determination of the biomass concentration difficult, so that the growth rates indicated in Table 2 may be questionable. The final biomass concentrations and yields were based on the total amount of biomass harvested at the end of the cultivation.

A cultivation profile of *M. circinelloides* on acetic acid is shown in Fig. 1. Exponential growth was evident up to 13.5 h, at which point total nitrogen depletion occurred. Thereafter, biomass accumulation and acetic acid assimilation proceeded at a significantly slower rate. Although there was only a relatively slight increase in the intracellular lipid content of the biomass, its GLA content increased markedly after nitrogen depletion. This, together with the increase in the biomass concentration, resulted in a large increase in the amount of intracellular GLA per volume of culture.

In batch cultures on glucose, *M. circinelloides* reached a higher biomass concentration within a shorter period of time than on acetic acid (Table 2), even though the amounts of the inocula were similar. Apart from that, the cultivation parameters were quite similar to those obtained with acetic acid as carbon substrate. These results indicate that the use of acetic acid as carbon feedstock for the production of fungal GLA is feasible.

The fatty acid and lipid fraction profile over time obtained with *M. circinelloides* is shown in Fig. 2. Towards the end of the cultivation the neutral lipid fraction increased to more than 90% of the oil, concomitant with a large decrease in the glycolipid fraction. The glycolipid and phospholipid fractions constituted 4.5% and 4.3%, respectively, of the oil. The fatty acid profiles of *M. circinelloides* and *M. rouxii* grown on acetic acid did not differ greatly. Oleic acid (18:1) was the most abundant fatty acid at about 34%, followed by palmitic acid (16:0) at about 23% of the total fatty acids.

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acetate is used as carbon source, independently of the kind of microorganism. Therefore, the content of D3 will suggest to the skilled person, when trying to keep the pH at a desired value for optimal production of DHA in response to an increase in the pH, to use a pH-stat mode in the culture of D1 where acetate is simultaneously used as titrant and carbon source. Therefore, the subject-matter of claims 4,5,7 is not inventive (Art. 33(3) PCT).

Arguments against the inventive step of the process claims 1-3, 28 and 29 can be raised also by combination of the disclosure of D1 and D2 since the acetic acid is an evident and cheaper alternative to glucose in fermentation cultures and it is well known from D1 that C. cohnii growth very well in acetate (see D1, p. 396, RH col. 4th paragrah).

The inventive merit of the subject-matter of claims 6,8-14,16, 17, 21-24 and 27 is not evident at present. See, for instance, pH ranges and yeast concentrations of D2 (col. 4, l. 55- col. 5, l. 22). Moreover, the optimisation of individual parameters seems to be within the customary practise of the skilled man.

Re Item VII
Certain defects in the international application

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1 and D3 is not mentioned in the description, nor are these documents identified therein.

Re Item VIII
Certain observations on the international application

1. The Applicant should note that the phrase "for the synthesis of docosahexaenoic acid by the microorganism" in claims 28 and "for the synthesis of polyunsaturated fatty acids by the microorganism" in claim 29 has no limitting effect on the methods proposed (PCT Guidelines, III, 4.8). On the other hand, the phrase "synthesising docosahexaenoic acid/polyunsaturated fatty acid" defines merely the result to be achieved without defining the technical features needed for achieving this result (Art. 6 PCT and PCT Guidelines, III, 4.7). The technical

features necessary for achieving this result have not been provided for claims 28 and 29.

2. It is doubtful that any microorganism can grow in presence of any acidic organic species as carbon source. The skilled person can think in many acidic organic compounds, for instance, proteins which contain carboxylic acids, antibiotics like penicillin or ampicillin, or other acidic organic species like benzoic acid, which can hardly be used as carbon source by any microorganisms, and, even more, these acidic organic compounds can even be toxic for it (refer to claims 28 and 29).

   Therefore, claim 28 and 29 does not fulfil the requirements of Art. 5 and 6 PCT due to the lack of limits of the technical features mentioned in said claims, ie microorganism and organic species, in order to secure the synthesis of DAH or other polyunsaturated fatty acids. These limits are also considered necessary to allow the skilled person to reduce the invention to practice without undue experimental efforts (Art. 5 PCT). In this respect, it is noted that the experimental data actually only refer (support-Art. 6 PCT) to six C. cohnii strains and acetic acid as carbon source and that the feature "synthesising docosahexaenoic acid/polyunsaturated fatty acid" is not considered to be able to overcome this objection.

3. It is doubtful that any "genetically modified variant" (claim 1) can produce DHA (Art. 6 PCT). On the other hand, it would appear that the subject-matter of claim 1 is not substantially supported in the description (Art. 6 PCT). Thus, only acetic acid/acetate has been shown as carbon source.

4. The term "about" used in claim 20 and 27 and throughout the description in connection with numerical values renders the scope of said claims unclear (Art. 6 PCT).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
10/W32314WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/02695	13/07/2000	14/07/1999
International Patent Classification (IPC) or national classification and IPC C12P7/64		
Applicant THE UNIVERSITY OF HULL		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 11 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12/01/2001	17.10.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Lopez Garcia, F Telephone No. +49 89 2399 2171

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/02695

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-23 as originally filed

Claims, No.:

1-33 with telefax of 31/08/2001

Drawings, sheets:

1/7-7/7 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☒ the claims, Nos.: 34-39

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 33.

because:

☒ the said international application, or the said claims Nos. with respect to industrial applicability relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

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☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4-14, 16, 17, 21-24, 27
	No:	Claims	1-3, 15, 18-20, 25, 26, 28-33
Inventive step (IS)	Yes:	Claims	
	No:	Claims	4-14, 16, 17, 21-24,27
Industrial applicability (IA)	Yes:	Claims	1-32
	No:	Claims	33

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item I

Basis of the report

The amendments filed with the fax dated 31.08.01 meet the requirements of Article 34(2)(b) PCT.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claim 33 relates to the use of a microorganism which produces docosahexaenoic acid (**DHA**) or a polyunsaturated fatty acid, as a food or food complement. It is known that DHA and other polyunsaturated fatty acids are important in the treatment of several diseases and infant development (see D1, p. 393, 1st paragraph of the Introduction). Therefore, said claim relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

For the assessment of the present claim 33 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item IV

Lack of unity of invention

In the light of the prior art disclosed in D1 and D2 (see below), the problem underlying the present application is the provision of alternative methods for the production of DHA or polyunsaturated fatty acids by a microorganism, compositions of said fatty acids, microorganisms used for their production and their use. The solution proposed to this problem disclosed and claimed in the present application consists in the methods of

claims 1, 28 and 29, the compositions of claims 25, 26, 30 and 31, the microorganisms used for the production of said fatty acids of claim 32 and the method of use of claim 33. Due to the fact that methods to obtain DHA or polyunsaturated fatty acids, their compositions, the microorganisms used for their preparation and uses are known and due to the fact that no further common technical features can be distinguished which, in the light of the prior art can be regarded as especial technical feature establishing novelty and contributing to the inventive step with respect to D1 and D2, the IPEA is of the opinion that there is not single inventive concept underlying the claimed inventions of the present application in the sense of Rule 13.1 PCT. Consequently the application lacks unity "a posteriori" and claims 1, 28, 29, 25, 26, 30, 31, 32 and 33 are considered as independent inventions.

The following 7 inventions are found:

a) Alternative methods of preparation of DHA (1 invention) 1. Claim 1-24, 27, 28 (all complete)

b) Compositions of DHA (2 inventions): 1. Claim 25 (complete), and claim 30 (insofar as DHA in concerned) 2. Claim 26 (complete) and claim 31 (insofar as DHA is concerned)

c) Alternative methods for the preparation of polyunsaturated fatty acids (1 invention): Claim 29 (insofar as DHA is not involved);

d) Compositions of polyunsaturated fatty acids (2 inventions): 1. Claim 30 (insofar as DHA in not involved) and 2. Claim 31 (insofar as DHA is not involved);

e) Microorganisms and used of said microorganisms (1 invention): 1. Claim 32 (complete) and claim 33 (complete).

However, and due to the amount of novelty objections, the lack of unity is, at present, not raised but the Applicant should bear in mind that in further examination stages or in the Regional Phase, if the unity defects still remain, that he will be requested to either restrict or subdivide the subject-matter of present application to the different inventions.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   D1: VAZHAPPILLY ET AL JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 75, 1998-03, p. 393-397.
   D2: US-A-5 407 957.
   D3: DU PREEZ ET AL BIOTECHNOLOGY LETTERS, vol. 17, 1995, p. 933-938.

2. The application discloses a method of culturing Crypthecodinium cohnii or variants thereof with carboxylic acids or carboxylate ions, for instance acetate, as a carbon source for the synthesis of docosahexaenoic acid (**DHA**).

3.1. D1 discloses a comparative study concerning DHA production of several microalga strains including C. cohnii. This microorganism showed good heterotrophic growth when acetate was used as carbon source (Table 1 at p. 394). The presence of other carbon sources will in principle not change this reasoning since other carbon sources are not excluded from claim 1 (see also claim 3) and the amount of acetate used in D1 (1gr/l and compare with the application p. 16, l. 14) seems to indicate that it is the main, if not the sole, carbon source (see also p. 396, RH col. 4th paragraph). Since the nature of the carbon source will presumably not change drastically the metabolism of C. cohnii, it is assumed that during this culture DHA is produced. The Applicant is drawn to the fact that omission of features (explicit mention of production of DHA), does not mean that they are not (implicitly) present (PCT Guidelines, IV, 7.5). Moreover, the microorganism C. cohnii and the carboxylic specie (acetic acid) seem to be the sole features needed for the synthesis of DHA, according to claim 1. Fish oil is known in the art as an alternative source of DHA (p. 393, LH col. last paragraph). Porphyridium medium seems to contain yeast extract. The subject-matter of claims 1-3, 15, 18-20, 28-32 is therefore not novel (Art. 33(2) PCT).

3.2. D2 discloses the production of a single cell edible oil containing high levels of DHA by a fermentation process using C. cohnii in presence of glucose as carbon

source (see Example and col. 5, l. 50-57). The application of DHA in food industry is known in the art (col. 6, l. 27). Alternative sources of DHA are also known (col. 1, l. 20-25, l. 62-63). Several microorganisms that produce DHA are also known (col 1, l. 34-43). Therefore, the subject-matter of claims 25, 26, 30-33 is not novel (Art. 33(2) PCT).

3.3. The Applicant should note that the products and uses of claims 25, 26, 30, 31, 32 and 33 (oil comprising DHA or polyunsaturated fatty acids, purified DHA or polyunsaturated fatty acids, the microorganisms used for the preparation of DHA or polyunsaturated fatty acids and the method of use of said DHA or polyunsaturated fatty acids) do not meet the requirements of Art. 33(2) PCT, since they are already known in the art (see 3.1 and 3.2 above) and they cannot be distinguished from those products and uses already known because of the method used to produce the products, the degree of purity or the use made of them.

3.4. Summing up, the subject-matter of claims 1-3,15,18-20,25,26,28-33 is not considered as novel (Art. 33(2) PCT).

4. D1, which is considered as the closest prior art, discloses the production of DHA by C. cohnii using acetate as carbon and energy source (p. 396, RH col. 4th paragraph).

The problem to be solved can be regarded as the provision of alternative methods for the production of DHA by C. cohnii.

The solution proposed, as far as it can be seen, differs from D1 by the simultaneous use of the carboxylic acid or carboxylate as titrant and carbon source in the method of production of fatty acids.

However, the solution is considered as not inventive since D3 already discloses a pH-stat mode culture using acetate as a titrant and carbon source (see "Abstract", p. 933 and "Material and Methods: Bioreactor cultivation", p. 934). Therefore, D3 already suggests the simultaneous use of acetate as tritant and carbon source. The method of D3 appears to be applicable to any fermentation procedure where

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/02695

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P7/64 C12N1/12 A23D9/00 C12M1/36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N A23D C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, PAJ, WPI Data, FSTA, CHEM ABS Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAZHAPPILLY REMA ET AL: "Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth." JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 75, no. 3, March 1998 (1998-03), pages 393-397, XP002152068 ISSN: 0003-021X	1-6, 20-39
Y	page 396, right-hand column; tables 1-3	7-19

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2000	22/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Devijver, K

4

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 00/02695

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	DU PREEZ J C ET AL: "Production of gamma-linolenic acid by Mucor circinelloides and Mucor rouxii with acetic acid as carbon substrate." BIOTECHNOLOGY LETTERS, vol. 17, no. 9, 1995, pages 933-938, XP000960382 ISSN: 0141-5492 page 933 -page 936; figures 1,2; table 2	7-19
A	US 5 407 957 A (KYLE DAVID J ET AL) 18 April 1995 (1995-04-18) cited in the application the whole document	1-39

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/02695

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5407957 A	18-04-1995	AT 165212 T	15-05-1998
		AU 660162 B	15-06-1995
		AU 7330291 A	03-09-1991
		BR 9106038 A	02-02-1993
		CA 2076018 A	14-08-1991
		DE 69129296 D	28-05-1998
		DE 69129296 T	05-11-1998
		DK 515460 T	22-02-1999
		EP 0515460 A	02-12-1992
		ES 2116288 T	16-07-1998
		IL 97126 A	15-03-1995
		IL 111174 A	10-03-1998
		JP 2830951 B	02-12-1998
		JP 5503425 T	10-06-1993
		WO 9111918 A	22-08-1991
		US 5492938 A	20-02-1996
		US 5711983 A	27-01-1998
		US 5397591 A	14-03-1995

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:
MATHISEN, MACARA & CO.
The Coach House
6-8 Swakeleys Road, Ickenham
UXBRIDGE, Middlesex UB10 8BZ
UNITED KINGDOM

COMMUNICATION IN CASES FOR WHICH
NO OTHER FORM IS APPLICABLE

Applicant's or agent's file reference 10/W32314WO	Date of mailing *(day/month/year)* 14/12/2000
International application No. PCT/GB 00/02695	**REPLY DUE** See paragraph 1 below
Applicant THE UNIVERSITY OF HULL	International filing date *(day/month/year)* 13/07/2000

1. ☐ REPLY DUE within _____ ~~months~~/days from the above date of mailing

   ☐ NO REPLY DUE

2. COMMUNICATION:

Further to your letter of 041200 - ref. 10/SJ/W32314WO - , we are enclosing a corrected version of form PCT/ISA/210 (first sheet) on which it appears that the title has been approved

A copy of this letter and its enclosure have been sent to the International Bureau of WIPO.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mireille Claudepierre

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
10/W32314WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/02695	13/07/2000	14/07/1999
Applicant		
THE UNIVERSITY OF HULL		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 2

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

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CLAIMS

1. A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing the microorganism with an organic species comprising an acidic group or an ionised form of an acidic group, the microorganism using the species as a carbon source and synthesising docosahexaenoic acid.

2. A method according to claim 1, wherein the microorganism is a dinoflagellate or a genetically modified variant thereof.

3. A method according to claim 2, wherein the microorganism is *Crypthecodinium cohnii* or a genetically modified variant thereof.

4. A method according to any one of claims 1 to 3, wherein the species is a carboxylic acid or a carboxylate ion.

5. A method according to claim 4, wherein the species is acetic acid or acetate.

6. A method according to any one of claims 1 to 5, wherein the species is the main carbon source for the microorganism during the culture of the microorganism.

7. A method according to any one of claims 1 to 6, wherein the microorganism

is cultured in a medium, an amount of the species being provided in the medium over a period of time during the culture of the microorganism.

8. A method according to claim 7, wherein the use of the species as a carbon source by the microorganism causes an increase in pH of the medium, said provision of the amount of the species comprising addition of an organic acid to the medium in response to the increase in pH so as to decrease the pH of the medium.

9. A method according to claim 8, wherein the organic acid is the species.

10. A method according to claim 8, wherein the organic acid ionises to form the species.

11. A method according to any one of claims 8 to 10, wherein the organic acid is added so as to maintain the pH substantially at a desired value.

12. A method according to claim 11, wherein the desired value is pH 6.5.

13. A method according to any one of claims 8 to 12, wherein the pH of the medium is monitored by means that produces a signal that is used to control the addition of the organic acid to the medium.

14. A method according to claim 13, wherein the signal is used to control addition of one or more of a nitrogen source, a phosphorus source, an amino acid, a vitamin, a salt or another growth factor during the culture of the microorganism.

15. A method according to any one of claims 8 to 13, wherein the organic acid is added to the medium in a mixture comprising a further compound.

16. A method according to claim 15, wherein the further compound is a further organic acid.

17. A method according to claim 15, wherein the further compound is a lipid.

18. A method according to any one of claims 15 to 17, wherein the mixture is a waste product from an industrial process.

19. A method according to claim 15, wherein the further compound is a nitrogen source, a phosphorus source, an amino acid, a vitamin, a growth factor, a salt or a lipid.

20. A method according to any one of claims 1 to 19, wherein prior to said culture with said species, the microorganism is grown with said species.

21. A method according to any one of claims 1 to 20, wherein the microorganism is cultured with an organic nitrogen source, preferably with yeast extract.

22. A method according to claim 21, wherein the nitrogen source is yeast extract and the initial concentration of the yeast extract is greater than 7.5 g/l.

23. A method according to claim 22, wherein the initial concentration of yeast extract is 10 g/l.

24. A method according to any one of claims 1 to 23, wherein the microorganism is cultured with salts or osmoticants, preferably with sea salts.

25. A method according to any one of claims 1 to 24, wherein said culture is performed as a batch process or a fed-batch process.

26. A method according to claim 25, wherein the culture is performed for between about 4 to about 10 days, preferably between about 6 to about 9 days.

27. A method according to any one of claims 1 to 25, wherein said culture is performed as a continuous process or semi-continuous process.

28. A method according to any one of claims 1 to 27, wherein the method further

comprises extracting oil including docosahexaenoic acid from the microorganism and, preferably, purifying the oil to increase the docosahexaenoic acid content of the oil.

29. A method according to any one of claims 1 to 28, wherein the method further comprises the purification or partial purification of docosahexaenoic acid from the microorganism.

30. A method according to any one of claims 1 to 29, wherein the culture does not include a stationary phase.

31. An oil comprising docosahexaenoic acid prepared from a microorganism cultured in accordance with any one of claims 1 to 30.

32. An at least partially purified preparation of docosahexaenoic acid prepared from a microorganism cultured in accordance with any one of claims 1 to 30.

33. A method according to claim 5 or any claim dependent on claim 5, wherein the initial concentration of the species is about 8 g/l.

34. A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing the microorganism with an organic species comprising an acidic group or an ionised form of an acidic group, the

microorganism synthesising docosahexaenoic acid containing carbon from the species.

35. A method of culturing a microorganism for the synthesis of a polyunsaturated fatty acid by the microorganism, comprising culturing *C. cohnii* with an organic species comprising an acidic group or an ionised form of an acidic group, the *C. cohnii* using the species as a carbon source and synthesising a polyunsaturated fatty acid.

36. An oil comprising said polyunsaturated fatty acid of claim 35, prepared from a microorganism cultured in accordance with claim 35.

37. An at least partially purified preparation of said polyunsaturated fatty acid of claim 35, prepared from a microorganism cultured in accordance with claim 35.

38. A microorganism cultured in accordance with any one of claims 1 to 30, 34 or 35.

39. A method comprising using a microorganism according to claim 38 as a food or a food supplement.